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14. ABSTRACT
Ebolavirus (EBOV) is a member of the filovirus family and causes severe hemorrhagic fever, resulting in death in up to 90% of infected humans. EBOV infection induces massive bystander lymphocyte apoptosis; however, neither the cellular apoptotic pathway(s) nor the systemic implications of lymphocyte apoptosis in EBOV infection are known. Herein we show that EBOV-induced lymphocyte apoptosis in vivo occurs via both the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, as both Fas associated death domain (FADD) dominant negative transgenic mice and mice overexpressing bcl-2 were resistant to EBOV-induced lymphocyte apoptosis. Surprisingly, inhibiting lymphocyte apoptosis during EBOV infection did not result in improved animal survival. Furthermore, we show for the first time that significant hepatocyte apoptosis likely occurs in EBOV infection, and that mice lacking the proapoptotic genes bim and bid had reduced hepatocyte apoptosis and marked reduction in liver enzyme levels in plasma after infection. Collectively, these data suggest that EBOV induces multiple pro-apoptotic stimuli and that blocking lymphocyte apoptosis is not sufficient to improve survival in EBOV infection. These data suggest that hepatocyte apoptosis may play a role in the pathogenesis of EBOV infection while lymphocyte apoptosis appears to be non-essential for EBOV pathogenesis.

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Mechanisms and Consequences of Ebolavirus-Induced Lymphocyte Apoptosis

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Ebolavirus (EBOV) is a member of the filovirus family and causes severe hemorrhagic fever, resulting in death in up to 90% of infected humans. EBOV infection induces massive bystander lymphocyte apoptosis; however, neither the cellular apoptotic pathway (s) nor the systemic implications of lymphocyte apoptosis in EBOV infection are known. In this study, we show data suggesting that EBOV-induced lymphocyte apoptosis in vivo occurs via both the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, as both Fas-associated death domain dominant negative transgenic mice and mice overexpressing bcl-2 were resistant to EBOV-induced lymphocyte apoptosis. Surprisingly, inhibiting lymphocyte apoptosis during EBOV infection did not result in improved animal survival. Furthermore, we show for the first time that hepatocyte apoptosis likely occurs in EBOV infection, and that mice lacking the proapoptotic genes *Bim* and *Bid* had reduced hepatocyte apoptosis and liver enzyme levels postinfection. Collectively, these data suggest that EBOV induces multiple proapoptotic stimuli and that blocking lymphocyte apoptosis is not sufficient to improve survival in EBOV infection. These data suggest that hepatocyte apoptosis may play a role in the pathogenesis of EBOV infection, whereas lymphocyte apoptosis appears to be nonessential for EBOV disease progression. ***The Journal of Immunology*****, 2010, 184: 327–335.**

Ebolavirus (EBOV) causes acute hemorrhagic fever and has a lethality rate of 35–90% in humans. The disease is characterized by high viral titers, fever, liver dysfunction, and coagulopathy [reviewed in (1)]. A prominent finding in lethal EBOV infection is extensive lymphocyte apoptosis, which is observed in vivo in mice, nonhuman primates, and likely in humans (2–7). Originally, it was hypothesized that EBOV-induced lymphocyte apoptosis abrogated the ability of the innate and adaptive immune system to respond to infection (5, 6). However, recent studies have indicated that a functional $CD8^+$ T cell-mediated immune response is generated in lethal EBOV infection in mice, but likely is outpaced by the rapid replication of the virus (8). Currently, it is not known what impact lymphocyte apoptosis has on the quality and magnitude of the immune response to EBOV, or whether inhibition of apoptosis would be beneficial to the host.

Apoptosis can proceed through two main pathways: the extrinsic (death receptor) pathway or the intrinsic (mitochondrial-mediated) pathway [reviewed in (9)]. In the extrinsic pathway, ligation of "death receptors," such as the Fas and TRAIL receptors, with their cognate ligands induces activation of the caspase-8 cascade through the adaptor protein Fas-associated death domain (FADD). In turn, the caspase cascade leads to DNA degradation and cell death. The extrinsic pathway can be blocked by inhibiting the binding of ligands to the death receptors, or by inhibition of death receptor signaling, for example, blockade/inactivation of FADD. In the intrinsic pathway, intracellular stress factors cause depolarization of the mitochondrial membrane, thereby inducing the release of cytochrome *c* and activating the caspase cascade beginning with caspase-9 (9). The intrinsic pathway can be inhibited by overexpression of Bcl-2, which stabilizes the mitochondrial membrane and prevents the release of cytochrome *c*. In addition, crosstalk occurs between these two pathways. Once activated, caspase-8 can cleave Bid into proapoptotic, truncated (t)Bid. tBid is a Bcl-2 antagonist and induces mitochondrial membrane permeabilization and activation of the intrinsic apoptotic pathway (9).

Lymphocytes are not infected by EBOV, but lymphocyte apoptosis is a prominent feature of infection. Therefore, it is hypothesized that lymphocyte apoptosis is caused by factors expressed or secreted by EBOV-infected cells such as macrophages or dendritic cells (5, 6, 10). This assertion is supported by the findings that EBOV-infected monocyte-like cells increase TRAIL expression in vitro, and that some EBOV-infected monkeys have an increase in soluble Fas in their sera (10). Furthermore, TRAIL and Fas mRNA expression is increased in the PBMC of infected monkeys (6). It has also been shown that a 17-mer peptide sequence in filovirus gps induces lymphocyte apoptosis in vitro through an unknown mechanism (11). However, no previous studies have analyzed the effect of blocking either the intrinsic or extrinsic apoptotic pathways to determine the mechanism of EBOV-induced lymphocyte apoptosis.

Another feature of EBOV infection is liver dysfunction. EBOV replicates to high titers in the liver (12–14), and fatal infection is

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Abbreviations used in this paper: ALT, alanine transaminase; AST, aminotransferase; EBOV, ebolavirus; FADD, Fas-associated death domain; FADD-dn, dominant negative mutant of FADD; LCMV, lymphocytic choriomeningitis virus; t, truncated.

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associated with alterations in circulating enzyme levels indicative of liver damage (15–17). Microscopic analysis of infected livers shows histopathologic lesions, including hepatocyte damage and death (14, 18, 19). However, previous studies have not described apoptosis as a mechanism of cell death in hepatocytes during EBOV infection.

Knowledge of whether EBOV-induced apoptosis contributes to disease-associated morbidity and/or mortality and identification of the particular cell death pathway(s) that are activated during EBOV infection could provide new insights into therapeutic approaches for this highly lethal pathogen. In this study, we used transgenic mice to show that lymphocyte apoptosis likely occurs via both the extrinsic and intrinsic apoptotic pathways during EBOV infection in vivo. However, inhibition of lymphocyte apoptosis did not improve animal survival, indicating that blocking lymphocyte apoptosis alone is not sufficient for controlling pathogenesis in EBOV infection. In addition, we demonstrate a role for hepatocyte apoptosis during EBOV infection. Mice lacking the proapoptotic proteins Bim and Bid have reduced hepatocyte apoptosis and improved liver function on day 5 after EBOV infection compared with wild-type mice. Together, these data demonstrate that EBOV infection causes significant apoptosis in multiple organs and that solely preventing this infection-associated apoptosis does not protect animals from the lethality of the infection.

Materials and Methods

Mice

All mice were on a C57BL/6 background. Vav-bcl–2 transgenic mice, in which Bcl-2 is overexpressed on all cells of hematopoietic origin (20), were a kind gift from Dr. Jerry Adams (Walter and Elisa Hall Institute, Melbourne, Australia). FADD dominant negative mice in which the FADD adaptor is inactive in T cells (21), and Bim/Bid null mice were provided by Dr. Andreas Strasser (Walter and Elisa Hall Institute, Melbourne, Australia). FasL null, TRAIL null, and TRAIL/gld null mice were kind gifts from Dr. Tom Ferguson, (Washington University School of Medicine, St. Louis, MO). Wild-type littermates were used in the majority of experiments and C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) were used when littermates were not available.

Ebola infections

Mice were infected with 1000 PFU of mouse-adapted EBOV-Zaire via i.p. injection (14). EBOV titers were determined using plaque assays on Vero cells. All EBOV-infected cells and mice were handled under maximum containment in a biosafety level-4 laboratory at the U.S. Army Medical Research Institute of Infectious Diseases (Frederick, MD). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Tissue processing

Mice were euthanized on day 0 or day 7 post-EBOV infection. Spleens, thymi, livers, and lymph nodes were harvested, fixed in 10% formalin, embedded in paraffin, and sectioned. Sections were stained with H&E or with a TUNEL kit (Chemicon, Temecula, CA) as per the manufacturer's instructions. Plasma or sera were collected from mice via intracardiac puncture and collection into EDTA or serum tubes. Measurement of aspartate aminotransferase (AST) and alanine transaminase (ALT) levels in sera or plasma were performed using Chem 13 reagent disks on a Piccolo analyzer (Abaxis, Union City, CA).

Transmission electron microscopy

For electron microscopic studies, samples were placed overnight in universal fixative (4% paraformaldehyde plus 1% glutaraldehyde in 0.1 mol/l Millonig's phosphate buffer) and were rinsed in Millonig's phosphate buffer. Samples were then immersed in 1% osmium tetroxide in 0.1 mol/l Millonig phosphate buffer. After rinsing, samples were stained with 0.5% uranyl acetate in ethanol, followed by dehydration in graded ethanol and propylene oxide. Specimens were then embedded in Poly/Bed 812 resin (Polysciences, Warrington, PA). Ultrathin sections were cut from 1-mm toluidine blue-stained sections and were placed on 200-mesh copper grids for transmission electron microscopy. After staining with uranyl acetate and lead citrate, the sections were analyzed with a JEOL transmission electron microscope (JEOL, Tokyo, Japan).

Examination of apoptosis in tissue sections via conventional brightfield microscopy and TUNEL staining

Tissue sections from spleens, thymi, lymph nodes, and livers from uninfected or Ebola-infected mice were stained by H&E and examined by brightfield microscopy by an investigator who was blinded to sample identity. A minimum of 5–6 random fields (magnification ×200) were evaluated and representative fields photographed. In addition to brightfield microscopy of H&E stained specimens, evaluation of DNA strand breaks, a classic finding in apoptosis, was examined via TUNEL (Chemicon). Tissue sections that had TUNEL staining were evaluated in a similar fashion. Higher magnifications were used for finer evaluation of cell injury.

Flow cytometry

Splenocytes were isolated by passing spleens through a mesh filter. RBCs were lysed with RBC lysing buffer (Sigma-Aldrich, St. Louis, MO) and washed with RPMI 1640 medium or PBS containing 2% fetal calf serum. Abs (purchased from eBioscience, San Diego, CA) were added at 1:100 dilution, incubated for 15 min at 4°C and then washed. Clones used were GK1.5 (CD4), B220 (RA3–6B2), CD3 (145–2C11), CD8 (53–6.7), IFN-γ (XMG1.2), and CD44 (IM7).

TUNEL staining with analysis by flow cytometry was performed as follows. Splenocytes were stained with fluorescent Abs against CD3, CD19, and CD8, and then cells were fixed for 3 d in 10% formalin. Cells were permeablized with 500 ug/ml digitonin for 10 min. Then, TUNEL staining was carried out using a fluorescent TUNEL kit from Chemicon, according to the manufacturer's protocol. Samples were analyzed on a FACSCanto II (BD Biosciences, San Jose, CA). Cells were gated on forward and side scatter and populations were identified as follows: total lymphocytes (forward scatter low side scatter low), B cells ($CD19^+$ $CD3^-$), $CD8^+$ T cells ($CD3^+$ $CD8^+$), and $CD3^+$ $CD8^-$ cells (which consisted of $CD4^+$ T cells [data not shown]).

Intracellular cytokine staining

Intracellular cytokine staining was performed by incubating splenocytes with 1 ug/ml of either two EBOV peptides (RIGNQAFLQEFVLPP and AKPLRNIMYDHLPGF), known to be $CD8^+$ T cell epitopes in mice after EBOV infection [(8), and S. Bradfute and S. Bavari, unpublished observations], or an irrelevant MARV GP peptide (MRTTCLFISLILIQGI) as a negative control. Brefeldin A was added and cells were incubated for 4–5 h before staining with CD3 and CD8. Cells were then washed, fixed, and permeabilized using the BD cytofix/cytoperm kit according to the manufacturer's instructions (BD Biosciences), and stained for intracellular IFN-γ. Cells were analyzed with a FACSCanto II (BD Biosciences). Cells were gated on forward and side scatter, and $CD3^+$ $CD8^+$ cells were analyzed for IFN-γ production.

Results

Lymphocyte apoptosis in mice after EBOV infection

Wild-type mice were infected with mouse-adapted EBOV (14), which typically causes death 7–10 d postinfection. On day 7, spleens, lymph nodes, and thymi from control or infected wild-type mice were harvested, fixed, stained, and analyzed for lymphocyte apoptosis. As shown in Fig. 1*A*, H&E staining revealed features of lymphocyte apoptosis including compacted, pyknotic nuclei and nuclear fragmentation (karyorrhexis) in spleens from day 7 infected mice, but not in control uninfected (day 0) spleens. In addition, analysis of spleen and thymus sections demonstrated depletion of cellular elements consistent with loss of lymphocytes; this loss was present in the white pulp of the spleen. TUNEL staining labels DNA strand breaks, a hallmark of apoptosis. Examination of TUNEL-stained spleen (Fig. 1*B*), thymi, and lymph node (Supplemental Fig. 1) sections showed that day 7 EBOV-infected tissues, but not day 0 tissues, had high levels of TUNEL-positive cells. Furthermore, electron microscopy examination of day 7 spleen samples revealed the presence of apoptotic nuclei in lymphocytes, as evidenced by compacted nuclear chromatin (Fig. 1*C*).

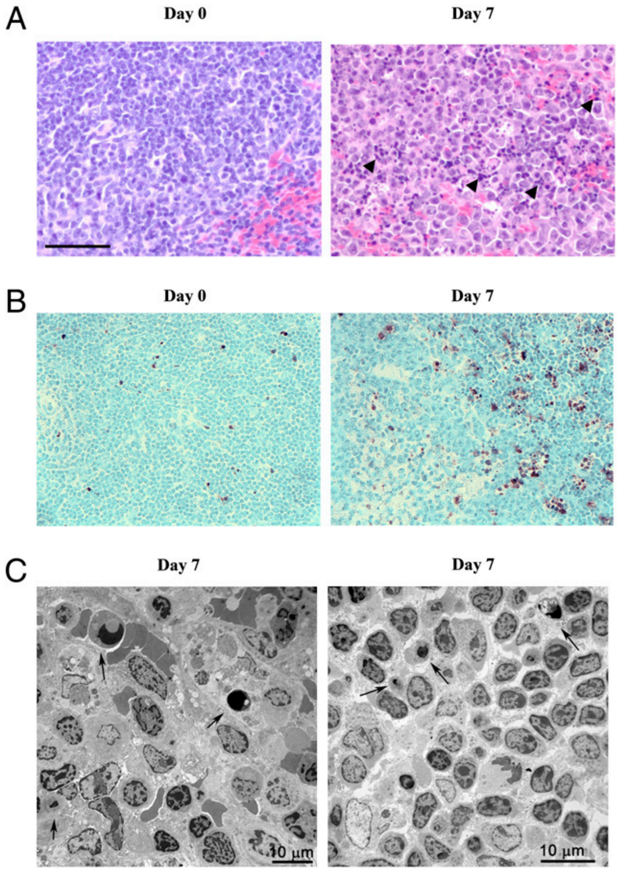

FIGURE 1. Lymphocyte apoptosis in the mouse model of EBOV infection. *A*, Lymphocyte apoptosis is evident in spleens from day 7 EBOV-infected, but not uninfected mice. Condensed, pyknotic nuclei characteristic of apoptosis is seen in day 7 mice H&E sections (arrowheads). Scale bar is 100 μm. *B*, TUNEL staining confirms apoptosis in spleen of infected mice. Counterstain is methylene green. *C*, Electron micrographs of spleen from Ebola-infected mouse with multiple apoptotic lymphoid cells. *Left panel*: Crescent-shaped chromatin diagnostic of apoptosis is present in upper left (arrow). Two other apoptotic cells with compacted chromatin are identified by arrow. (Original magnification ×2500.) *Right panel*: Additional apoptotic splenocytes identified by compacted chromatin. Note the lack of necrotic properties such as cell swelling and membrane rupture. Original magnification ×2500.

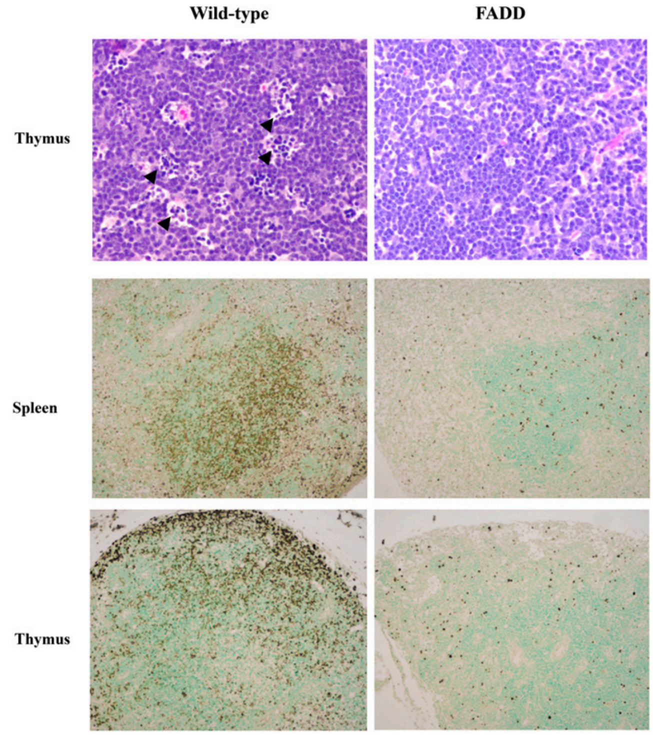

FIGURE 2. FADD dominant negative mice have decreased lymphocyte apoptosis following EBOV infection. Thymi and spleens from wild-type or FADD-dn mice that express a dominant negative mutation of FADD in T cells were infected with EBOV and analyzed on day 7. H&E and TUNEL staining reveal significant protection from lymphocyte apoptosis in FADD-dn mice relative to wild-type mice. TUNEL counterstain is methylene green. Arrowheads highlight apoptotic cells in H&E sections. Original magnification ×200.

Together with previously published reports, these data strongly support the use of the mouse model as a template for studying the role of lymphocyte apoptosis in EBOV pathogenesis (4, 8).

The role of death receptor signaling in EBOV-induced lymphocyte apoptosis

To determine which apoptosis pathway(s) are involved in EBOV-induced lymphocyte death, we infected several groups of transgenic mice defective in different apoptosis pathways. Because all known death receptors use FADD to transmit their apoptotic signal, we analyzed mice carrying a dominant negative mutant of FADD (FADD-dn) in T cells to study the role of the extrinsic apoptotic pathway in EBOV-induced lymphocyte apoptosis. On day 7 postinfection, spleens, thymi, and lymph nodes were examined for evidence of lymphocyte apoptosis. FADD-dn mice showed a lesser degree of lymphocyte apoptosis after EBOV infection compared with wild-type littermate controls, as demonstrated by H&E or TUNEL staining of thymus and spleens (Fig. 2). Quantitation of apoptosis by software-based determination of TUNEL staining in tissue sections of spleen and thymi similarly showed a moderate reduction in the number of apoptotic cells (data not shown). To determine whether TRAIL or Fas pathways were involved, mice lacking either TRAIL, Fas, or both TRAIL and FasL (TRAIL-gld) were infected with EBOV, and spleens were analyzed by TUNEL staining. As shown in Supplemental Fig. 2*A*, there was no noticeable visual difference in apoptosis in these mice compared with wild-type mice. We confirmed this finding by performing quantitation of TUNEL staining in the spleen sections (Supplemental Fig. 2*B*) and, in a separate experiment, by analyzing TUNEL staining in splenic lymphocytes by flow cytometry (Supplemental Fig. 2*C*).

The role of the intrinsic apoptotic pathway in EBOV-induced lymphocyte apoptosis

To determine whether the intrinsic apoptosis pathway is involved in EBOV-induced lymphocyte death, we infected mice lacking *Bim* and *Bid* proapoptotic genes involved in the intrinsic apoptotic pathway. Bim/Bid knockout mice were resistant to lymphocyte apoptosis after EBOV infection (Supplemental Fig. 3). To further analyze the role of the intrinsic apoptotic pathway in EBOV-induced lymphocyte apoptosis, we infected mice overexpressing bcl-2 in all hematopoietic cells (transgenic bcl2 expression was under control of the vav promoter) (22). Vav-bcl–2 mice showed nearly complete protection against lymphocyte apoptosis compared with wild-type littermate control mice (Fig. 3). This finding was confirmed by flow cytometric evaluation of TUNEL-stained splenocytes from a different cohort of EBOV-infected mice. Total lymphocytes, as well as individual T and B cell subsets, all had substantially decreased apoptosis in vav-bcl–2 mice relative to wild-type mice 7 d after EBOV infection (Fig. 4*A*, 4*B*). Lymphocytes in day 7 vav-bcl–2 mice had

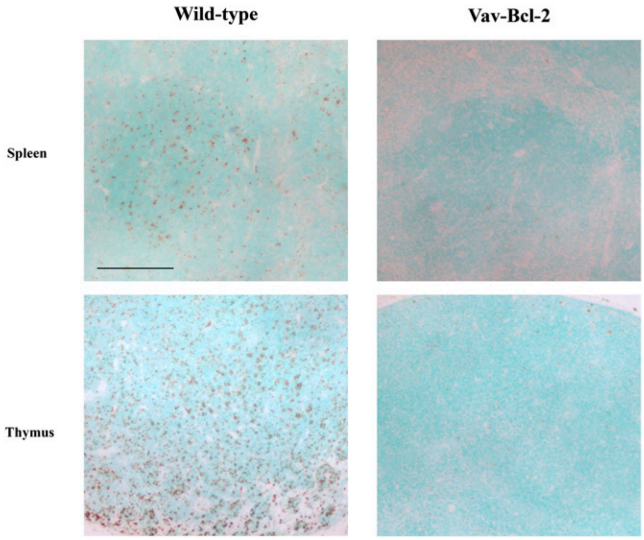

FIGURE 3. Overexpression of Bcl-2 protects lymphocytes from EBOV-induced apoptosis. TUNEL staining on thymi and spleens from day 7 EBOV-infected wild-type or vav-bcl–2 transgenic mice. Drastically reduced apoptosis is observed in lymphocytes overexpressing bcl-2. Counterstain is methylene green. Scale bar is 100 μm. Original magnification ×200.

such low TUNEL staining levels that they were similar to control samples that were mock-stained (i.e., no TdT enzyme added to the staining) (Fig. 4*A*).

Inhibition of lymphocyte apoptosis does not alter mortality or time to death in EBOV-infection

To determine whether lymphocyte apoptosis plays a role in the lethality of EBOV, survival experiments were performed. Neither vav-bcl–2 mice nor FADD-dn mice had increased survival compared with wild-type mice after EBOV infection (Fig. 5). In addition, time-to-death was similar in all groups. This is noteworthy, because it has been hypothesized that lymphocyte apoptosis is a key event in the pathogenesis of EBOV infection (6). We also analyzed the possibility that inhibition of lymphocyte apoptosis, though not sufficient to improve animal survival, might be partially protective in controlling EBOV replication. However, viremia in day 7 sera was increased in vav-bcl–2 mice compared with wild-type littermates (Supplemental Fig. 4*A*). We have previously shown that a functional, EBOV-specific $CD8^+$ T cell response is generated in lethally infected mice (8). To determine whether inhibition of lymphocyte apoptosis resulted in increased $CD8^+$ T cell response to EBOV infection, we analyzed the production of IFN-γ by day 7 $CD8^+$ T cells in response to EBOV peptides. As shown in Supplemental Fig. 4*B*, both wild-type and vav-bcl–2 mice produced increased IFN-γ in response to these particular EBOV peptides compared with control Marburg virus peptide. However, wild-type mice had a more robust $CD8^+$ T cell response than vav-bcl–2 mice, suggesting that inhibition of apoptosis did not increase $CD8^+$ T cell function in vav-bcl–2 mice.

Indication of EBOV-induced hepatocyte apoptosis

During our analysis of a variety of tissues in EBOV-infected mice, we observed pervasive liver damage. Severe liver dysfunction is a hallmark of lethal EBOV infection, as evidenced by hepatocyte death and accumulation of the liver enzymes AST and ALT in the blood (15–17). Hepatocyte necrosis has been described in EBOV infection and may be a direct consequence of extensive viral replication in these cells (6, 23–25). However, apoptotic cell death of hepatocytes in EBOV-infected mice has not been reported. H&E and TUNEL staining in the liver have been used to study hepatocyte apoptosis in other models (26, 27). We analyzed the livers of EBOV-infected mice to determine whether hepatocyte apoptosis was present. Indeed, hepatocytes in day 7 EBOV-infected mice exhibited hallmarks of apoptosis, including pyknotic nuclei (Fig. 6*A*) and positive staining in the TUNEL assay (Fig. 6*B*). Although TUNEL staining can produce false positives, hepatic sections from EBOV-infected mice that were TUNEL positive also had secondary morphologic features of apoptosis, including fragmented and compacted nuclei. Furthermore, electron microscopic analysis of livers from EBOV-infected mice appeared to identify apoptotic hepatocytes (Fig. 6*C*, 6*D*). This evidence suggests that hepatocyte apoptosis occurs during EBOV infection. Necrotic hepatocytes were also observed (data not shown), confirming earlier reports (6, 23–25).

Mice deficient in the proapoptotic proteins Bim and Bid reduce EBOV-induced liver dysfunction

Liver damage is thought to contribute to EBOV pathogenesis and may be central to man sequelae of infection (1). Therefore, to determine whether hepatocyte apoptosis can be inhibited, mice lacking the proapoptotic genes *Bim* and *Bid* were infected with EBOV. Bim/Bid mice had decreased hepatocyte apoptosis compared with wild-type mice 7 d postinfection (Fig. 7*A*, 7*B*, Supplemental Fig. 5). More importantly, the levels of AST and ALT were reduced in Bim/Bid-deficient mice on day 5 compared with wild-type mice (Fig. 7*C*), although this reduction was not observed on day 7 (data not shown). However, Bim/Bid mice were not protected against lethality after EBOV infection (Fig. 7*D*); this correlates with the lack of AST and ALT reduction on day 7 (data not shown). Nonetheless, these findings raise the possibility that ameliorating hepatocyte damage in EBOV infection is feasible and may be useful as an adjunct therapy to treat the disease.

Discussion

Lymphocyte apoptosis is a characteristic of many viral infections. For example, infection of mice with an H1N1 strain of influenza results in lethal disease course that induces apoptosis of influenza-specific $CD8^+$ T cells via the Fas/FasL pathway (28). Mice lacking FasL survive infection, suggesting that inhibition of $CD8^+$ T cell apoptosis reverses pathogenesis of influenza infection. On the other hand, mice infected with an acute strain of lymphocytic choriomeningitis virus (LCMV) demonstrate lymphocyte apoptosis at days 3 and 8 postinfection; however, they generate a robust adaptive immune response and clear the infection (29–32). Therefore, the importance of lymphocyte apoptosis in viral infections must be determined experimentally for each individual disease.

We have demonstrated that, similar to what is seen in human and nonhuman primate EBOV infections, extensive lymphocyte apoptosis occurs in the mouse model of EBOV infection [Fig. 1, (4, 8)]. Previous studies have shown that human PBMCs or monocyte-like cells infected with EBOV in vitro upregulate TRAIL and Fas/FasL and downregulate bcl-2 postinfection, suggesting a role of these proteins in EBOV-induced lymphocyte apoptosis (10, 33). However, no studies have blocked these pathways to tease out the mechanisms of apoptosis. To better understand EBOV pathogenesis, we used transgenic mice defective in various apoptotic pathways. Our data demonstrate that functional loss of the scaffolding protein FADD, which is used by all death receptors, conferred partial rescue of lymphocytes from EBOV-induced apoptosis (Fig. 2). However, a lack of TRAIL, Fas, or both FasL and TRAIL did not inhibit lymphocyte apoptosis (Supplemental Fig. 2), suggesting that multiple death receptor pathways are involved. A similar finding was reported in a model of sepsis (34). Although the TRAIL and Fas pathways are not required for lymphocyte apoptosis

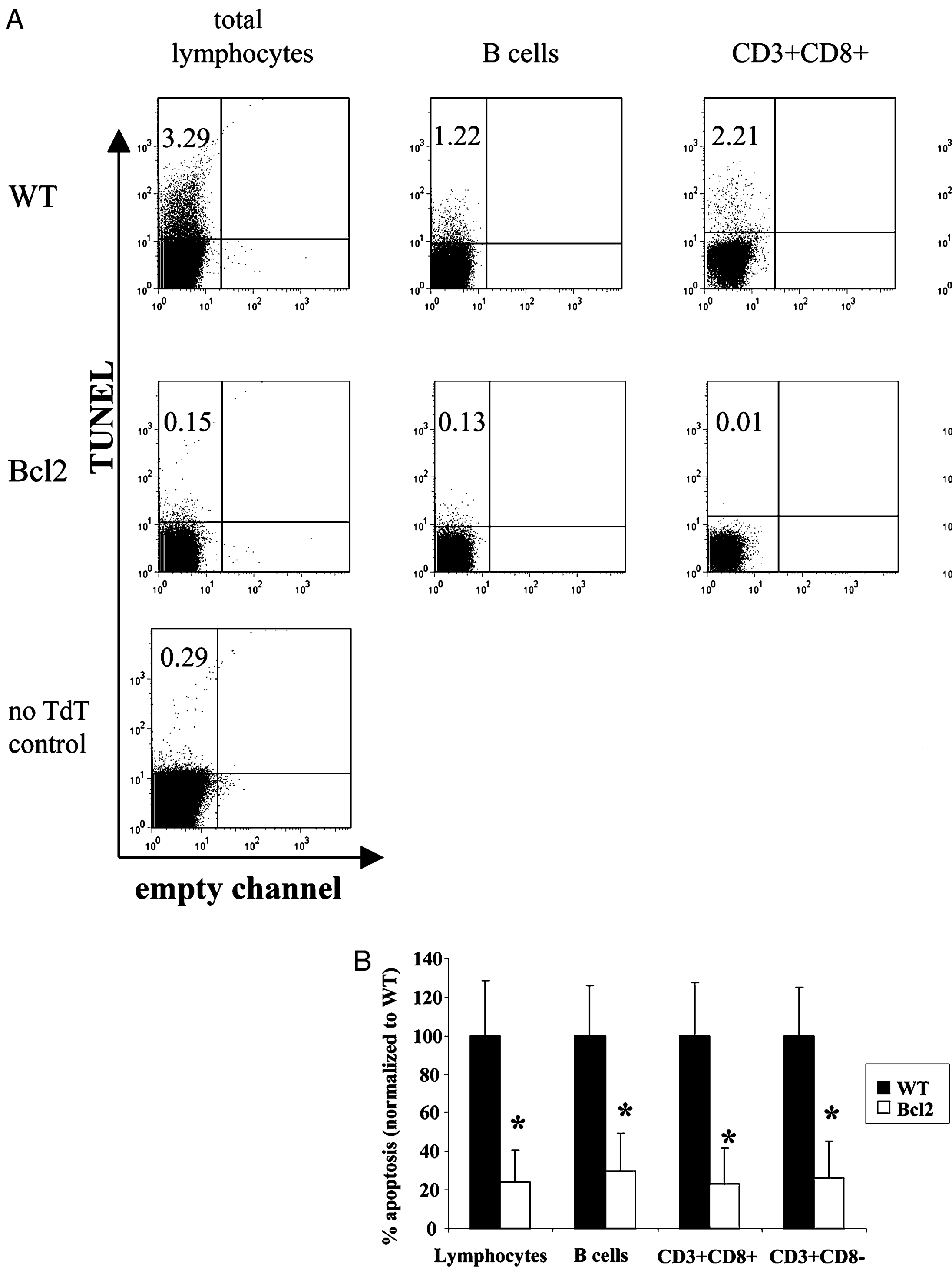

FIGURE 4. Quantitation of reduced apoptosis in vav-bcl–2 mice. *A*, Splenocytes from wild-type or vav-bcl–2 mice were stained with Abs against CD19 (B cells), CD3, and CD4. Cells were then fixed, stained with a fluorescent TUNEL kit and analyzed with flow cytometry. A remarkable decrease in apoptosis was evident in all lymphocyte subsets analyzed from vav-bcl–2 mice relative to control wild-type mice. The relatively low percentage of apoptotic cells in day 7 wild-type mice is likely a byproduct of the harsh fixation conditions used to bring cells out of biosafety level-4 conditions; day 0 samples show ~1% TUNEL positive cells (data not shown), instead of the 3–5% staining generally reported, suggesting that the baseline for TUNEL staining after these fixation conditions is lowered. *B*, Data were averaged and normalized to wild-type levels of apoptosis. $n = 7$ for wild-type and $n = 8$ for vav-bcl–2. $*p < 0.05$.

in EBOV infection, it is possible that they are used to induce lymphocyte apoptosis, but that other FADD-dependent pathways are also induced. In addition, mice overexpressing bcl-2 in hematopoietic cells had near-complete protection against lymphocyte apoptosis after EBOV infection (Figs. 3, 4).

These results suggest that both the intrinsic and extrinsic apoptotic pathways are used in EBOV-induced lymphocyte apoptosis, but that inhibiting either pathway reduces apoptosis (summarized in Fig. 8). In light of the partial protection conferred by preventing death receptor mediated signaling, it was surprising to find that transgenic overexpression of bcl-2 in all hematopoietic cells conferred near complete protection from EBOV-induced lymphocyte apoptosis. There are at least three explanations for the protection from EBOV-induced lymphocyte apoptosis in vav-bcl2 mice. In the

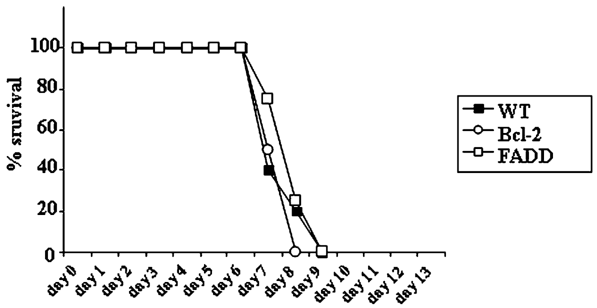

FIGURE 5. Inhibition of lymphocyte apoptosis does not confer animal survival or control of viral replication. Wild-type, vav-bcl–2, or FADD-dn mice were infected with 1000 PFU of EBOV. All animals succumbed to infection by day 9. $n = 8–10$ per group.

first instance, it is possible that after induction of the extrinsic death receptor pathway, caspase-8 does not efficiently activate caspase-3, but instead cleaves Bid to tBid, thereby "crossing over" to the intrinsic pathway. This would then be blocked by overexpression of bcl-2. In the second instance, it could be that supraphysiologic bcl2 expression in the vav-bcl2 mice has secondary effects on cellular physiology that contravene death receptor signaling. Alternately, it appears increasingly likely that there is more extensive crosstalk between the intrinsic and extrinsic pathways than previously thought. It has been proposed that active caspase-3 can, in a retrograde manner, activate both caspase-8 and caspase-9, thereby connecting both the intrinsic and extrinsic systems (35). It can be argued that our results do not conclusively demonstrate that the intrinsic pathway is activated independent of the extrinsic pathway. However, the only partial protection found in the thymi of EBOV-infected FADD-dn mice suggests that the intrinsic pathway is involved independently of the extrinsic pathway. Further experiments should shed light on this question.

Understandably, it has been widely proposed that lymphocyte apoptosis is an important event in EBOV pathogenesis and that preventing lymphocyte apoptosis could potentiate the immune response to EBOV, conferring protection from disease (3, 6, 36). However, the data reported in this study suggest that inhibition of lymphocyte apoptosis alone does not significantly alter EBOV lethality or diminish viral replication. A recent study from our laboratory demonstrated that despite bystander lymphocyte apoptosis, there is a functional EBOV-specific $CD8^+$ T cell response in lethally infected mice, and transfer of these cells can protect naive recipient mice from lethal EBOV infection (8). This immune response correlated with a sharp increase in peripheral lymphocyte numbers, increased CD44 expression, and the appearance of lymphoblasts; these characteristics are also found in EBOV infection of nonhuman primates, suggesting that this is not a mouse-specific phenomenon (6, 37). Therefore, EBOV-induced lymphocyte apoptosis does not eliminate the development of adaptive immune responses. Because only a fraction of T or B cells are specific for any given viral Ag, elimination of nonspecific lymphocytes would not necessarily diminish a specific response. It is possible that lymphocyte apoptosis is a common sequela of severe acute infection rather than a cause; supporting this assertion, we have recently found that transgenic mice resistant to EBOV infection have marked reduction in lymphocyte apoptosis (38). Lymphocyte apoptosis is a common finding in many other hemorrhagic fever viruses, including Lassa, Marburg, Crimean Congo hemorrhagic fever, and some Hantavirus infections. However, no studies to our knowledge have studied the impact of inhibiting lymphocyte apoptosis in these systems, so the physiologic relevance of these findings is unknown. In an LCMV murine model, lymphocyte apoptosis occurs in both Ag-specific and nonspecific $CD8^+$ T cells (29). It has been hypothesized that this apoptosis may "clear" space for increased specific T cell responses (31). However, the tools to study this question in the EBOV system are lacking, so these studies are outside the realm of our current investigations.

Although it has been hypothesized that lymphocyte apoptosis inhibits the development of a successful immune response to EBOV challenge (3, 6, 36), viral titers were actually higher in vav-bcl–2 mice compared with wild-type mice (Supplemental Fig. 4*A*). In addition, we found that there was decreased EBOV-specific $CD8^+$ T cell IFN-γ responses in vav-bcl–2 mice compared with wild-type mice (Supplemental Fig. 4*B*). Combined with the lack of animal survival, these data suggest that the immune response in mice resistant to EBOV-induced lymphocyte apoptosis is impaired compared with wild-type mice. It can be argued that because vav-bcl–2 mice have altered lymphocyte development due to increased cell

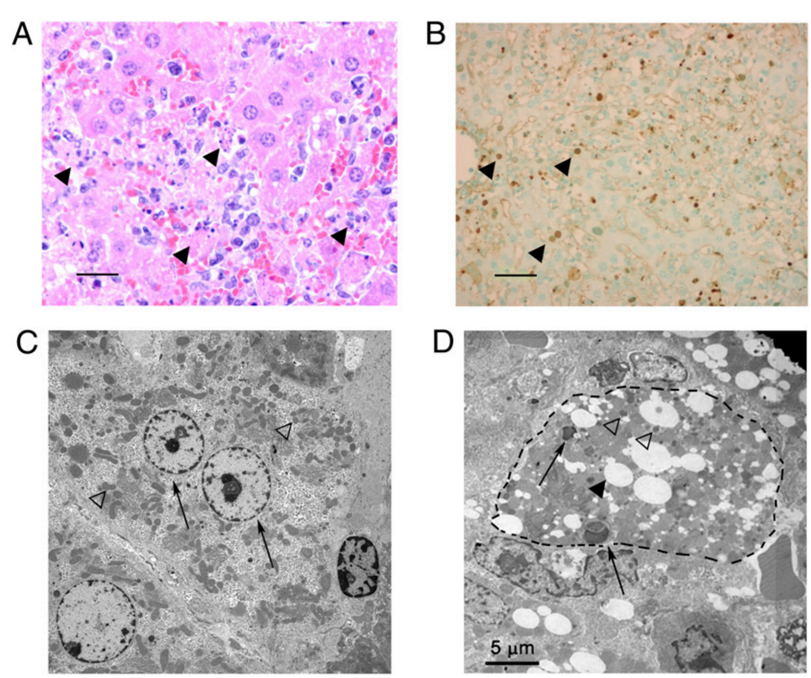

FIGURE 6. Hepatocyte apoptosis in EBOV infection. *A*, H&E section of a liver in a day-7 EBOV-infected mouse. Arrowheads point to pyknotic hepatocyte nuclei characteristic of classical apoptosis. Scale bar is 100 μm. *B*, TUNEL staining reveals apoptosis in hepatocytes (arrowheads). Counterstain is methylene green. Note the binuclear hepatocyte positive for TUNEL staining in the bottom left. Scale bar is 100 μm. C, Liver from uninfected mouse. Arrows point to healthy nuclei in a binucleated hepatocyte; empty arrowheads show mitochondria. Original magnification ×2500. *D*, Liver from Ebola-infected mouse. Shown is a hepatocyte containing apoptotic nuclear fragments, identified by arrows. Note the classical crescent-shaped nuclear fragment (lower part of cell), and compacted nuclear fragments. Lipid droplets are present (solid arrowhead), indicative of a hepatocyte. Dashed lines outline cell membrane. Original magnification ×2500.

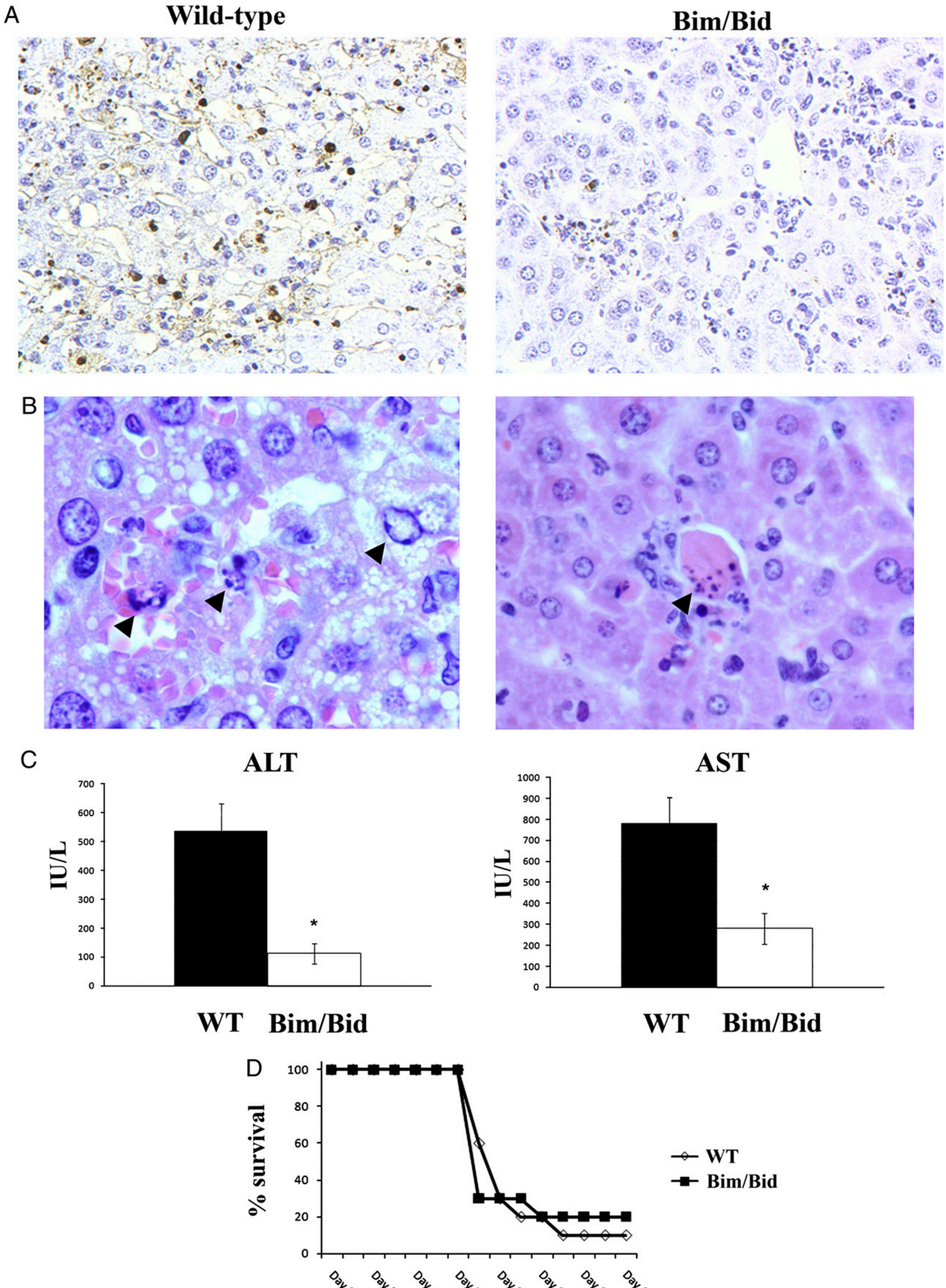

FIGURE 7. Bim/Bid knockout mice have delayed liver damage after EBOV infection. *A*, Decreased TUNEL staining is seen in Bim/Bid knockout mice relative to wild-type mice on day 7 postinfection. Counterstain is H&E. Original magnification ×200. *B*, H&E staining shows apoptotic hepatocytes in wild-type mice (arrowheads), with decreased apoptotic hepatocytes in Bim/Bid knockout mice. Original magnification ×200. *C*, Decreased ALT and AST levels are present in Bim/Bid knockout mice on day 5 of infection, suggesting preservation of liver function in these mice. n = 6–7 for wild-type, n = 7–8 for Bim/Bid. $*p < 0.01$. *D*, Bim/Bid knockout mice do not show increased survival after EBOV infection (n =10).

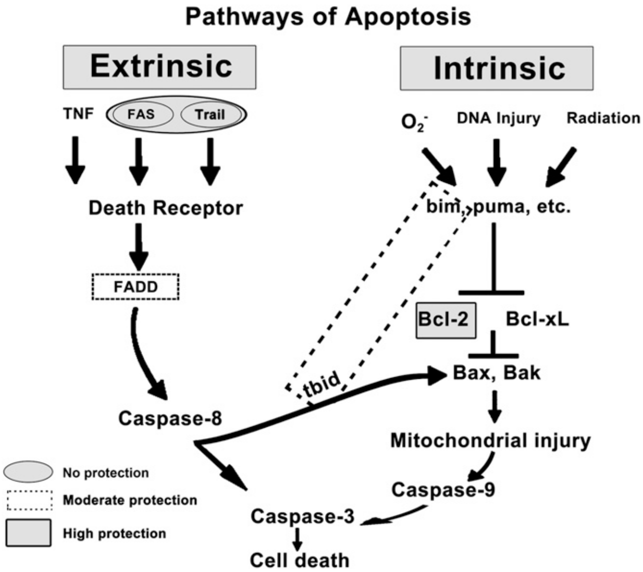

FIGURE 8. Summary of pathways used in EBOV-induced lymphocyte apoptosis. Both extrinsic and intrinsic pathways may be used in EBOV infection to induce lymphocyte apoptosis. Moderate protection was found in mice lacking FADD signaling or Bim and Bid, whereas near-complete protection was exhibited in mice overexpressing Bcl-2 in WBCs. It is important to note that this model is a generalization of apoptotic pathways; pathways for apoptosis will vary according to cell type.

survival (39), they are not functional in EBOV infection. However, we found significant EBOV-specific IFN-γ responses in $CD8^+$ T cells from day 7 vav-bcl–2 mice, albeit reduced compared with wild-type mice (Supplemental Fig. 4*B*). In addition, bcl-2–overexpressing mice are resistant to septic peritonitis-induced mortality, suggesting that the transgene does not render mice incapable of mounting an immune response [(40), data not shown]. Furthermore, vav-bcl–2 and Bim/Bid mice have been shown to produce high levels of specific Ab after vaccination (41), and FADD-DN mice are resistant to septic shock-induced death, suggesting that the immune response in these mice is not impaired (34). The vav-bcl–2 findings may be supported by alternative explanations. Bcl-2 overexpression may enhance the survival of regulatory T cells (42) that could have an adverse effect on the immune response to EBOV, or vav-bcl–2 overexpression may prevent macrophage and dendritic cell apoptosis which provides more target cells for EBOV replication. In addition, lymphocyte apoptosis may be beneficial to the generation of an immune response, as has been hypothesized in LCMV infection (29).

We have also demonstrated that apoptosis is likely to be responsible for at least a portion of the hepatocyte death that is commonly seen in EBOV infection (Fig. 6). Because the liver is a target organ for EBOV and the related Marburg virus, it is surprising that no published studies have focused on treatments to augment liver function in filovirus infection. We report in this study that Bim/Bid knockout mice have decreased hepatocyte apoptosis after EBOV infection compared with wild-type mice (Fig. 7, Supplementary Fig. 5). We also observed delayed liver dysfunction in Bim/Bid mice compared with wild-type mice as assessed by measurement of circulating AST and ALT levels (Fig. 7*C*). As therapeutics are pursued for filovirus infections, these findings suggest that including a treatment designed to stabilize liver function would be a rational adjunctive treatment for filoviral infection.

Though oft-maligned (43), the mouse model of EBOV infection has demonstrated mechanistic aspects of EBOV-mediated pathogenesis that would be intractable in primate models or observational clinical studies. In this study, we have taken advantage of a spectrum of transgenic and knockout mice to probe the mechanisms of cell death that occur in both hepatocytes and lymphocytes during EBOV infection and our results have strong correlates with empirical studies in humans and nonhuman primates. Although the mouse model for EBOV infection does not completely recapitulate the hemorrhagic clinical presentation in humans and nonhuman primates, particularly in a lack of fibrin deposition (7, 14), it has served a valuable role in testing vaccines and therapeutics, as well as providing a controlled in vivo environment for basic research regarding the pathogenesis of EBOV infection (4, 8, 44–52).

Overall, these data show that lymphocyte apoptosis in EBOV infection in vivo proceeds through both the intrinsic and extrinsic pathways. Simply blocking lymphocyte apoptosis does not protect mice from EBOV; however, combination therapies targeting multiple aspects of EBOV disease may be helpful in combating filovirus infection.

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Disclosures

The authors have no financial conflicts of interest.

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